University of Zurich[UZH]

Zurich Open Repository and Archive
University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2016

Arabidopsis NAC016 promotes chlorophyll breakdown by directly upregulating STAYGREEN1 transcription

Sakuraba, Yasuhito ; Han, Su-Hyun ; Lee, Sang-Hwa ; Hörtensteiner, Stefan ; Paek, Nam-Chon

Abstract: The Arabidopsis transcriptional factor NAC016 directly activates chlorophyll degradation during leaf senescence by binding to the promoter of SGR1 and upregulating its transcription. During leaf senescence or abiotic stress in Arabidopsis thaliana, STAYGREEN1 (SGR1) promotes chlorophyll (Chl) degradation, acting with Chl catabolic enzymes, but the mechanism regulating SGR1 transcription remains largely unknown. Here, we show that the Arabidopsis senescence-associated NAC transcription factor NAC016 directly activates SGR1 transcription. Under senescence-promoting conditions, the expression of SGR1 was downregulated in nac016-1 mutants and upregulated in NAC016-overexpressing (NAC016-OX) plants. By yeast one-hybrid and chromatin immunoprecipitation assays, we found that NAC016 directly binds to the SGR1 promoter, which contains the NAC016-specific binding motif (termed the NAC016BM). Furthermore, nac016-1 SGR1-OX plants showed an early leaf yellowing phenotype, similar to SGR1-OX plants, confirming that NAC016 directly activates SGR1 expression in the leaf senescence regulatory cascade. Although we found that NAC016 activates SGR1 expression in senescing leaves, this transcriptional regulation is considerably weaker in maturing seeds; the seeds of sgr1-1 mutants (also known as nonyellowing1-1, nye1-1) stayed green, while the seeds of nac016-1 mutants turned from green to yellow normally. We also found that the abscisic acid (ABA) signaling-related transcription factor genes ABI5 and EEL and the ABA biosynthesis gene AAO3, which activate SGR1 expression directly or indirectly, were significantly downregulated in nac016-1 mutants and upregulated in NAC016-OX plants. However, the NAC016BM does not exist in their promoter regions, indicating that NAC016 may indirectly activate these ABA signaling and biosynthesis genes, probably by directly activating transcriptional cascades regulated by the NAC transcription factor NAP. The NAC016-mediated regulatory cascades of SGR1 and other Chl degradation-related genes are discussed.

DOI: https://doi.org/10.1007/s00299-015-1876-8

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-130380
Journal Article
Accepted Version

Originally published at:
Sakuraba, Yasuhito; Han, Su-Hyun; Lee, Sang-Hwa; Hörtensteiner, Stefan; Paek, Nam-Chon (2016). Arabidopsis NAC016 promotes chlorophyll breakdown by directly upregulating STAYGREEN1 transcription. Plant Cell Reports, 35(1):155-166.
DOI: https://doi.org/10.1007/s00299-015-1876-8

Arabidopsis NAC016 promotes chlorophyll breakdown by directly upregulating *STAYGREEN1* transcription

Yasuhito Sakuraba[1], Su-Hyun Han[1], Sang-Hwa Lee[1†], Stefan Hörtensteiner[2], Nam-Chon Paek[1*]

[1] Department of Plant Science, Plant Genomics and Breeding Institute, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, 08826, Korea

[2] Institute of Plant Biology, University of Zurich, CH-8008 Zurich, Switzerland

*Corresponding author: Email: ncpaek@snu.ac.kr; Tel:+82-2-880-4543; Fax: +82-2-877-4550.

[†]Present address: CKD Research Institute, Yongin, 16995, Korea

Abstract

During leaf senescence or abiotic stress in *Arabidopsis thaliana*, *STAYGREEN1* (*SGR1*) promotes chlorophyll (Chl) degradation, acting with Chl catabolic enzymes (CCEs), but the mechanism regulating *SGR1* transcription remains largely unknown. Here we show that the *Arabidopsis* senescence-associated NAC transcription factor NAC016 directly activates *SGR1* transcription. Under senescence-promoting conditions, the expression of *SGR1* was downregulated in *nac016-1* mutants and upregulated in *NAC016*-overexpressing (*NAC016*-OX) plants. By yeast one-hybrid and chromatin immunoprecipitation assays, we found that NAC016 directly binds to the *SGR1* promoter, which contains the NAC016-specific binding motif (termed the NAC016BM). Furthermore, *nac016-1 SGR1*-OX plants showed an early leaf yellowing phenotype, similar to *SGR1*-OX plants, confirming that NAC016 directly activates *SGR1* expression in the leaf senescence regulatory cascade. Although we found that NAC016 activates *SGR1* expression in senescing leaves, this transcriptional regulation is considerably weaker in maturing seeds; the seeds of *sgr1-1* mutants (also known as *nonyellowing1-1*, *nye1-1*) stayed green, while the seeds of *nac016-1* mutants turned from green to yellow normally. We also found that the abscisic acid (ABA) signaling-related transcription factor genes *ABI5* and *EEL* and the ABA biosynthesis gene *AAO3*, which activate *SGR1* expression directly or indirectly, were significantly downregulated in *nac016-1* mutants and upregulated in *NAC016*-OX plants. However, the NAC016BM does not exist in their promoter regions, indicating that NAC016 may indirectly activate these ABA signaling and biosynthesis genes, probably by directly activating transcriptional cascades regulated by the NAC

transcription factor NAP. The NAC016-mediated regulatory cascades of *SGR1* and other Chl degradation-related genes are discussed.

Keywords: *Arabidopsis thaliana*, Leaf senescence, Chlorophyll degradation, *STAYGREEN1*, NAC016, Transcriptional regulation.

Key message

The *Arabidopsis* transcriptional factor NAC016 directly activates chlorophyll degradation during leaf senescence by binding to the promoter of *SGR1* and upregulating its transcription.

Introduction

Chlorophyll harvests light and transfers excitation energy to other components of the photosynthetic electron transport chain (Grossman et al., 1995). During senescence, Chl is degraded to colorless breakdown compounds in a multistep catabolic pathway, called the PAO/phyllobilin pathway, which comprises several chloroplast-located reactions (Hörtensteiner, 2013). To date, six Chl catabolic enzymes (CCEs), including Chl *b* reductases encoded by *NON-YELLOW COLORING1* (*NYC1*, Kusaba et al., 2007; Horie et al., 2009) and *NYC1-LIKE* (*NOL*, Sato et al., 2009; Horie et al., 2009), 7-hydroxymethyl Chl reductase (HCAR, Meguro et al., 2011), pheophytinase (PPH, Schelbert et al., 2009), pheophorbide *a* oxygenase (PAO, Pružinská et al., 2003), and red Chl catabolite reductase (RCCR, Pružinská et al., 2007) have been identified and characterized in *Arabidopsis* and rice.

In addition to CCEs, STAYGREEN (SGR) homologs function as key regulators of Chl degradation. The *Arabidopsis thaliana* genome contains three *SGR* homologs, named *SGR1* (termed *nonyellowing1* [*nye1*] or *no chlorosis1* [*noc1*] in *Arabidopsis sgr1* mutants), *SGR2*, and *SGR-LIKE* (*SGRL*), and they contribute differently to Chl degradation (Sakuraba et al., 2015a). Among the three *SGR* homologs, *SGR1* has been studied widely; during natural senescence and dark-induced senescence (DIS), the *nye1-1/sgr1-1* mutants in *Arabidopsis* showed a stay-green phenotype, while *SGR1*-OX plants showed an early leaf yellowing phenotype (Ren et al., 2007; Sakuraba et al., 2012). *nye1-1/sgr1-1* and *noc1-1/sgr1-2* mutants also sustained leaf greenness much more than WT under abiotic and biotic stress conditions at vegetative stages (Mecey et al.,

2011; Sakuraba et al., 2014a) and during the seed degreening stage (Delmas et al., 2013), indicating that SGR1 has an important role in activating Chl degradation throughout plant development. Similar to SGR1, SGRL also promotes Chl degradation, but this function is limited to stress-induced leaf yellowing during vegetative growth (Sakuraba et al., 2014b). All land plants have at least one SGR1 and one SGRL homolog (Barry et al., 2008; Sakuraba et al., 2014a), indicating that SGR1 and SGRL are essential for Chl degradation. Interestingly, *Arabidopsis* SGR2 and SGR1 have opposite functions despite their high amino acid sequence similarity (Sakuraba et al., 2014a). *SGR2*-OX plants showed a stay-green phenotype while *sgr2* knockout mutants showed early leaf yellowing during natural senescence and artificially induced senescence, thus indicating that SGR2 acts a negative regulator of Chl degradation during leaf senescence (Sakuraba et al., 2014a). We previously proposed that differences in their capacities to interact with CCEs cause the divergent functions of three SGR homologs (Sakuraba et al., 2015a). SGR1 and SGRL can interact with six CCEs (NYC1, NOL, HCAR, PPH, PAO, and RCCR) and the light-harvesting complexes II (LHCII; Sakuraba et al., 2012, 2013, 2014b). Although SGR2 also interacts with LHCII proteins, it hardly interacts with CCEs (Sakuraba et al., 2014a), leading to the interruption of formation of SGR1-CCE or SGRL-CCE complexes at LHCII.

Though the physiological and biochemical functions of SGR homologs and CCEs have been well studied during last decade, studies of the transcriptional regulatory cascades of *SGR* and CCE genes are still limited. We previously reported that NAC016, a *Arabidopsis* senescence-associated NAC (senNAC) transcription factor (TF), promotes leaf senescence; *nac016* KO mutants exhibited delayed senescence, while *NAC016*-OX plants exhibited accelerated leaf senescence under senescence-promoting conditions (Kim et al., 2013). NAC016 also has an important role in the drought stress-responsive pathway (Sakuraba et al., 2015b). Furthermore, we also identified the NAC016-specific binding motif (hereafter NAC016BM) by yeast one-hybrid and ChIP assays (Sakuraba et al., 2015b). Genome-wide gene expression profiling and promoter binding analyses revealed that the NAC016BM is enriched in NAC016-regulated genes and showed that NAC016 activates *NAP* (also known as *NAC029*), another *Arabidopsis* senNAC TF gene (Guo and Gan, 2006) by directly binding to its promoter region. Like *nac016* mutants, *nap* mutants showed delayed leaf senescence (Guo and Gan, 2006), and exhibited a tolerant phenotype under drought stress (Sakuraba et al., 2015b), indicating that NAP has an important role in leaf senescence and drought stress-responsive pathways, acting downstream of NAC016. Although we previously found the regulatory module of NAC016-NAP, it is possible that NAC016 also regulates other senescence-associated genes (SAGs) in the leaf senescence pathway. Indeed, a few senTFs regulate more

than two SAGs during leaf senescence (Rauf et al., 2013; Sakuraba et al., 2014c; Garapati et al., 2015).

Here we found that *SGR1* is a direct target of NAC016. Expression analysis revealed that the expression of *SGR1* was strongly repressed in *nac016-1* mutants during leaf senescence. Furthermore, promoter binding assays showed that NAC016 strongly interacts with the promoter of *SGR1*, which contains the NAC016BM. However, *SGR1* was not strongly repressed in *nac016-1* seeds; thus Chl degradation occurred normally in the maturing seeds of *nac016-1* mutants, unlike *nye1-1/sgr1-1* seeds. The transcriptional regulatory module of NAC016-SGR1 during leaf senescence is discussed.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (Col-0 ecotype) plants were grown on soil at 22-24°C under long-day (LD) conditions (16 h light:8 h dark). The *nac016-1* (SALK_001597C) mutant seeds were obtained from the Arabidopsis Biological Resource Center. The *35S:SGR1* (*SGR1*-OX), *35S:GFP-NAC016*, and *35S:NAC016* (*NAC016*-OX) plants were prepared previously (Sakuraba et al., 2012; Kim et al., 2013; Sakuraba et al., 2015b). The *nac016-1 SGR1*-OX line was prepared by crossing *nac016-1* and *SGR1*-OX plants.

Dark-induced senescence

For dark-induced senescence, detached rosette leaves (3rd or 4th leaves) or whole plants grown for 3 weeks under LD conditions were transferred to complete darkness (22°C). After dark incubation, the rosette leaves were sampled under safe green light.

Stress treatments

For the salt and osmotic stress assays, the 3rd or 4th rosette leaves of 3-week-old plants grown under LD conditions were incubated at 22°C on 3 mM MES (pH 5.8) buffer supplemented with 100 mM NaCl or 400 mM mannitol. For drought stress assays, 3-week-old plants grown under LD conditions were dehydrated on soil or on dry filter paper at 22°C.

Total Chl Measurement

To measure total Chl, photosynthetic pigments were extracted from the leaf tissues with 80% ice-cold acetone. Chl concentrations were determined using a UV/VIS spectrophotometer as described previously (Lichtenthaler, 1987).

Seed Phenotyping

Seed phenotype was observed using an inverted light microscope (Axio Observer Z.1, Carl ZEISS, Germany). Chl autofluorescence of seeds was observed using a laser scanning confocal microscope (SP8X STED, Leica, Germany).

RT-PCR and qPCR Analysis

For RT-PCR analysis, total RNA was isolated from the rosette leaf tissues using the TRIzol Reagent according to the manufacturer's protocol (Invitrogen). The first-strand cDNAs were prepared with 5 μg of total RNA in a 25 μl reaction volume using the M-MLV reverse transcriptase and oligo(dT)$_{15}$ primer (Promega), and diluted with water to 100 μl. The 20 μl of qPCR mixture contained 1 μl of cDNA template, 10 μl of 2X SYBR Green PCR Master Mix (Qiagen), and 0.25 mM of the forward and reverse primers for each gene. Primer sequences for each gene are listed in Supplemental Table S1. qPCR was performed using the Light Cycler 480 (Roche Diagnostics). To obtain the relative expression levels, mRNA levels of each gene were normalized to that of glyceraldehyde phosphate dehydrogenase (*GAPDH*).

Yeast One-Hybrid Assays

The *NAC016* cDNA was inserted into the pGAD424 vector (Clontech) as prey. DNA fragments corresponding to the promoters (-1500 to -751, and -750 to -1 from start codon) of *SGR1*, *SGR2*, and *NYC1* were cloned into the pLacZi (Clontech) as baits. Primers used for cloning are listed in Supplemental Table S1. The yeast strain YM4271 was used for bait and prey clones. β-Galactosidase activity (1 unit = 10^4 min^{-1} ml^{-1}) was measured by liquid assay using chlorophenol red-β-D-galactopyranoside (CPRG; Roche Applied Science) according to the Yeast Protocol Handbook (Clontech).

Chromatin Immunoprecipitation (ChIP) Assays

The *35S:GFP-NAC016* and WT plants were grown for 14 days under LD conditions, and dark-incubated for 2 days before cross-linking for 20 min with 1% formaldehyde under vacuum. Chromatin complexes were isolated and sonicated as previously described (Saleh et al., 2008) with slight modification. Anti-GFP polyclonal antibody (Abcam) and Protein A agarose/salmon sperm

DNA (Millipore) were used for immunoprecipitation. After reverse cross-linking and protein digestion, DNA was purified by QIAquick PCR Purification Kit (Qiagen). Primer sequences for each gene are listed in Supplemental Table S1.

Results

NAC016 activates CCE genes during DIS

To examine the regulatory relationship between the NAC016 senTF and expression of Chl degradation-related genes, we first examined the expression levels of three *SGR* genes (*SGR1/NYE1/NOC1*, *SGR2*, and *SGRL*) and six Chl degradation enzymes (*NYC1*, *NOL*, *HCAR*, *PPH*, *PAO*, and *RCCR*) in *nac016-1* mutants (Kim et al., 2013) during DIS. Similar to *NAC016*, the expression levels of *NYC1*, *PPH*, *PAO*, *SGR1*, and *SGR2* increased during DIS, and these genes were significantly downregulated in *nac016-1* mutants after 4 days of dark incubation (4 DDI; Fig. 1). Among these, *SGR1* expression was strongly repressed even before DIS (Fig. 1g), as previously shown by microarray analysis (Sakuraba et al., 2015b), suggesting the strong NAC016-*SGR1* transcriptional cascade. On the other hand, the expression levels of *NOL*, *HCAR*, *RCCR*, and *SGRL* were not significantly altered (Fig. 1).

NAC016 directly activates *SGR1* transcription during leaf senescence

We subsequently examined whether these Chl degradation-related genes contain the NAC016BM (TTGGATXXA) in their promoter regions; among these 9 Chl degradation-related genes, the *SGR1*, *SGR2*, and *NYC1* promoters have one NAC016BM each (Fig. 2a), indicating that NAC016 may directly target these three genes during senescence. However, *SGRL* and five other CCE genes (*NOL, HCAR, PPH, PAO*, and *RCCR*) do not contain the NAC016BM in their promoter regions. To examine whether NAC016 binds to the promoters of *SGR1*, *SGR2*, and *NYC1*, we performed yeast one-hybrid assays. To this end, we prepared two DNA fragments (-1500 to -751 and -750 to -1) of each promoter. We found that NAC016 binds strongly to the *SGR1* promoter, and interacts weakly but significantly with the promoters of *SGR2* and *NYC1* (Fig. 2b). To confirm NAC016 binding to these promoters *in vivo*, we performed ChIP assays. For the ChIP assay, we used the previously prepared *35S:GFP-NAC016* plants (Sakuraba et al., 2015b). The 2-week-old WT and *GFP-NAC016* plants were dark-incubated for 2 days. We found that in the dark-incubated leaves, GFP-NAC016 strongly binds to the amplicon-e of the *SGR1* promoter that contains the NAC016BM (Fig. 2c), similar to the results of the yeast one-hybrid assay (Fig. 2b). However, NAC016 did not bind to the promoters of *SGR2* or *NYC1* in spite of the presence of the

NAC016BM (Fig. 2c), indicating that NAC016 does not directly target *SGR2* and *NYC1 in planta*. Taken together these results indicate that among the Chl degradation-related genes, only *SGR1* is directly regulated by NAC016.

To examine the transcriptional cascade of NAC016-SGR1 in more detail, we measured the expression of *SGR1* in *nac016-1* mutants and *NAC016*-OX plants in three different senescence-promoting conditions. In addition to DIS and natural senescence conditions, we also used salt stress because we previously found that both *nac016-1* and *nye1-1/sgr1-1* mutants stayed green under salt stress conditions (Kim et al., 2013; Sakuraba et al., 2014a). Compared with WT, the expression of *SGR1* decreased in *nac016-1* mutants, and increased in *NAC016*-OX plants during DIS, natural senescence, and salt stress (Fig. 3). We also measured the expression of *NAC016* in *nye1-1* mutants under the three senescence-inducing conditions, but found that *NAC016* expression did not change in *nye1-1* mutants during senescence (Supplementary Fig. S1). Furthermore, we prepared *nac016-1 SGR1*-OX double mutant plants to examine the genetic epistasis between *NAC016* and *SGR1*. The *nac016-1 SGR1*-OX plants showed an early senescence phenotype during DIS with lower levels of total Chl, similar to *SGR1*-OX plants (Fig. 4a, b). These results confirm that *SGR1* acts downstream of NAC016 in leaf senescence.

Indeed, *nac016-1* and *nye1-1* mutants showed similar leaf stay-green phenotypes during DIS, natural senescence, and abiotic stresses, such as salt and osmotic stresses (Supplementary Fig. S2) (Ren et al., 2007; Kim et al., 2013; Sakuraba et al., 2014a), indicating that the NAC016-SGR1 regulatory module is important for age-dependent and stress-induced leaf yellowing. In addition, we recently revealed that NAC016 is required for the induction of drought stress responses, as *nac016* null mutants are tolerant and *NAC016*-OX plants are sensitive to drought stress (Sakuraba et al. 2015b). Furthermore, *SGR1* expression is downregulated in *nac016* mutants under drought stress conditions (Sakuraba et al. 2015b). In this study, we examined whether *sgr1-1/nye1-1* mutants or *SGR1*-OX plants are tolerant or susceptible to drought stress. The drought stress assay was performed by progressive dehydration on soil or more rapid dehydration on dry filter paper. Under both conditions, however, *nye1-1* and *SGR1*-OX plants showed neither tolerance nor sensitivity and became wilted similar to WT (Supplementary Fig. S3), indicating that *SGR1* is not related to the drought tolerance in *nac016* mutants or the hypersensitivity to dehydration in *NAC016*-OX plants.

NAC016 does not regulate *SGR1* transcription during seed degreening

A previous study reported that *nye1-1* mutants produce green seeds because Chl degradation is impaired during seed maturation (Delmas et al., 2013), indicating that *SGR1* has an important role

in seed degreening. To examine whether NAC016 directly regulates *SGR1* expression during seed greening, we examined the seed color of *nac016-1* mutants. We found that, strikingly, the *nac016-1* and *nye1-1* mutants had totally different phenotypes, as the *nac016-1* seeds turn from green to yellow, indicating Chl degradation occurs normally, as in WT seeds (Fig. 5a-c).

We also checked seed germination because Chl retention in mature seeds causes a decrease in germination rate (Parcy et al., 1997; Nakajima et al., 2012). As reported, the germination rate of *nye1-1* mutants was significantly lower than WT, but the germination rate of *nac016-1* seeds was almost the same as WT (Fig. 5d). Nakajima et al. (2012) also reported that the *Arabidopsis nyc1-1 nol-1* double mutants, having no Chl *b* reductase activity, have green-colored cotyledon in matured seeds and young seedlings exhibit a pale green phenotype with albino cotyledon. Although the exact mechanisms of pale-green seedling and white-cotyledon phenotypes in *nyc1-1 nol-1* double mutants are still unclear, it was proposed that these defective phenotypes might result from the Chl retention during seed maturation (Nakajima et al., 2012). Here we found that a few of the *nye1-1* mutants also showed both pale green and white-cotyledon phenotypes (Fig. 5e), similar to the *nyc1-1 nol-1* double mutants. By contrast, the cotyledons of *nac016-1* mutants showed a normal green color.

To further examine the regulatory relationship of NAC016-*SGR1* in seed development, we checked the expression levels of *SGR1* in the maturing seeds of WT and *nac016-1*. Although *SGR1* expression was slightly downregulated in *nac016-1* seeds, this regulation was negligible compared with *SGR1* down-regulation in leaves (Fig. 5f). These results indicate that NAC016 does not regulate *SGR1* expression during seed degreening.

NAC016 indirectly activates ABA synthesis and signaling pathways through the NAC016-NAP loop

We previously reported that NAC016 binds to the promoter of *NAP* and directly activates *NAP* expression during senescence and under drought stress (Kim et al., 2013; Sakuraba et al., 2015b). NAP directly activates the ABA biosynthesis gene *ABA ALDEHYDE OXIDASE3* (*AAO3*) (Yang et al., 2014) and the ABA signaling PP2C type TF gene *SENESCENCE-ASSOCIATED GENE113* (*SAG113*) (Zhang et al., 2012). Yang et al. (2014) also showed that Chl degradation-related genes, including *SGR1*, *NYC1*, *PPH*, and *PAO* are significantly downregulated in *nap* mutants, but the NAP senNAC TF does not bind to the promoters of these Chl degradation genes (Yang et al., 2014). This indicates that NAP indirectly promotes Chl degradation-related genes by activating ABA synthesis. Furthermore, we recently found that two ABA signaling-related TFs, ABA

INSENSITIVE5 (ABI5) and ENHANCED EM LEVEL (EEL), directly activate *SGR1* and *NYC1* expression during leaf senescence (Sakuraba et al., 2014c).

To examine the regulatory relationship between NAC016-SGR1 and other members of the *SGR1* regulatory cascade, we examined the expression levels of *NAP*, *AAO3*, *ABI5*, and *EEL* in WT, *nac016-1*, and *NAC016*-OX plants during DIS and natural senescence. The expression levels of *NAP*, *AAO3*, *ABI5*, and *EEL* were significantly downregulated in *nac016-1* mutants, and were upregulated in *NAC016*-OX plants (Fig. 6). The NAC016 TF directly binds to the NAP016BM in the *NAP* promoter (Sakuraba et al., 2015). We subsequently examined whether the three genes have the NAC016BM in the promoter regions, but found no sequence similarity to the NAC016BM. This indicates that NAC016 indirectly promotes the expression of *AAO3*, *ABI5*, and *EEL*, probably via the activation of *AAO3* expression by NAP (Yang et al., 2014).

We also checked the expression patterns of *NAC016* in response to exogenous treatment of ABA. Rosette leaves from 3-week-old WT plants were incubated in the MES buffer supplemented with 50 μM ABA for 24 hours. The expression levels of *NAC016* significantly increased during ABA treatment (Supplementary Fig. S4), indicating that NAC016 is an ABA-responsive gene like *NAP*, *AAO3*, *ABI5*, and *EEL*.

Discussion

In this study, we found that NAC016 directly targets *SGR1* under senescence-promoting conditions. *SGR1* is one of the best-studied SAGs in *Arabidopsis* (Sakuraba et al., 2015a) and *SGR1* homologs have been isolated and characterized in other plant species, including rice (Park et al., 2007), pea (Armstead et al., 2007), tomato (Barry et al., 2008), bell pepper (Barry et al., 2008), tall fescue (Wei et al., 2011), *Medicago truncatula* (Zhou et al., 2011), and soybean (Fang et al., 2014). Also, *sgr1* knockout mutants in these plant species commonly show a very strong stay-green phenotype during senescence, suggesting the vital role for *SGR1* in the leaf senescence pathway, including Chl degradation.

Most studies of *SGRs* and *CCEs* have focused on their biochemical and physiological functions (Hörtensteiner, 2013; Sakuraba et al., 2015a), but recent studies in *Arabidopsis* and rice identified several senTFs that directly promote expression of *SGRs* and *CCEs*. In rice, the OsNAP senNAC TF directly promotes expression of *SGR*, *NYC1*, *NYC3* (PPH homolog in rice), and *RCCR* (Liang et al., 2014). By contrast, *Arabidopsis* NAP indirectly promotes expression of *SGRs* and *CCEs* by directly activating the ABA synthesis gene *AAO3* (Yang et al., 2014). In addition, we previously showed that in *Arabidopsis*, two ABA bZIP TFs, ABI5 and EEL directly activate

expression of *SGR1* and *NYC1* during DIS (Sakuraba et al., 2014c). Based on these previous studies and our present results, we propose a model of NAC016-mediated regulation of *SGR1* and *NYC1* (Fig. 7). In this study, we found that NAC016 directly activates expression of *SGR1*, but not *NYC1* (Fig. 2b, c). Instead, NAC016 directly activates *NAP* expression (Kim et al., 2013), which probably leads to the activation of *AAO3* in the ABA biosynthetic pathway. Increased ABA activates expression of *ABI5* and *EEL* and, in turn, these proteins directly activate expression of *SGR1* and *NYC1*. In this study, we also revealed that ABA treatment activated *NAC016* expression (Supplementary Fig. S4), indicating that the NAC016-NAP-AAO3-ABA regulatory module forms a coherent feed-forward loop for activating leaf senescence, including Chl degradation. Similar feed-forward loops were found in the leaf senescence pathway of *Arabidopsis* (Kim et al., 2009; Sakuraba et al., 2014c), and may make the senescence pathway less prone to disruption by ephemeral environmental fluctuations (Le and Kwon, 2013).

In this study, we also found that *SGR2*, *NYC1*, *PPH*, and *PAO* were downregulated in *nac016-1* mutants during DIS (Fig. 1b). Using our proposed model (Fig. 3c), we show how NAC016 indirectly activates the expression of *NYC1* and other *CCEs*. Previous work reported that ABA activates the expression of *SGR1*, *NYC1*, *PAO*, and *PPH* in *Arabidopsis* rosette leaves (Yang et al., 2014). Thus, probably NAC016 indirectly activates *NYC1*, *PPH*, *PAO*, and *SGR2* expression by enhancing ABA synthesis and signaling during leaf senescence, and NAC016 thus regulates *SGR1* expression both directly and indirectly.

It is also possible that in addition to ABI5 and EEL, other ABA signal-related TFs directly regulate the expression of *SGR1* and other Chl degradation-related genes. During seed degreening, the ABA-related TF *ABA INSENSITIVE3* (*ABI3*) directly activates *SGR1* and *SGR2,* and *ABRE-BINDING FACTOR4* (*ABF4*) activates *NYC1* (Delmas et al., 2013; Nakajima et al., 2012). Thus, it is possible that several ABA signaling-related TFs induce expression of *SGR1* and Chl degradation-related genes during leaf senescence. Indeed, by checking expression patterns of ABA signaling TFs in the *Arabidopsis* eFP browser, we found that, on the one hand, several ABA signaling genes, including *ABF1, ABF3, ABF4, ABI1, ABI2, ABI5,* and *EEL* were highly expressed in senescing leaves, with expression patterns consistent with those *NAC016*, *NAP*, *SGR1*, and *NYC1* (Supplementary Fig. S5). On the other hand, the expression levels of *ABI3* and *ABI4* were lower in senescent leaves, but were high in maturating seeds, similar to *SGR1*, *NYC1*, *ABF4*, *ABI5*, and *EEL* (Supplementary Fig. S6), indicating that the transcriptional regulation of *SGR1* and *NYC1* by ABA signaling-related TFs somehow differs between leaves and seeds. We also found that the expression levels of *NAC016* increased in maturing seeds (Supplementary Fig. S5), like in senescing leaves (Supplementary Fig. S4) (Kim et al. 2013), indicating that NAC016 has an

important role in seed maturation. However, we found that the yellow seed phenotype of *nac016-1* mutants is quite different from the green-seed phenotype of *nye1-1* mutants, because *SGR1* expression for Chl degradation is not repressed in *nac016-1* seeds (Fig. 5). Thus, the contribution of NAC016 to *SGR1* activation differs in leaves and seeds. Further transcriptome and physiological analyses of the *nac016-1* seeds are needed to elucidate the difference.

Although we have identified two direct target genes of NAC016, *SGR1* and *NAP*, during leaf senescence (Fig. 2) (Kim et al. 2013), NAC016 might also directly regulate other SAGs using the NAC016BM. The NAC016BM differs from the binding motifs of other senNAC TFs, such as *ORESARA1* (*ORE1/NAC092*; Olsen et al., 2005), *NAP* (Zhang et al., 2012), *JUNGBRUNNEN1* (*JUB1/NAC042*; Wu et al., 2012), *ORESARA1 SISTER1* (*ORS1/NAC059*; Balazadeh et al., 2011), and *VND-INTERACTING2* (*VNI2/NAC083*; Yang et al., 2011). Furthermore, the NAC016BM does not contain the core motif CACG (or reverse complement CGTG) to which most NAC proteins bind (Sakuraba et al., 2015b). Thus, NAC016 probably regulates leaf senescence differently from other senNAC TFs. Moreover, further identification of direct NAC016-regulating SAGs will be important for elucidation of the transcriptional regulatory cascades in the leaf senescence pathway.

Author contribution statement

YS and NCP conceived and designed research, and wrote the manuscript. YS, SHH, and SHL conducted experiments. SH contributed new reagents or analytical tools. All authors read and approved the manuscript.

Acknowledgments

We thank to Prof. Benke Kuai for donating *nye1-1* seeds. This work was carried out with the support of “Cooperative Research Program for Agricultural Science & Technology Development (Project No. PJ01107901), Rural Development Administration, Republic of Korea.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

Armstead I, Donnison I, Aubry S, Harper J, Hörtensteiner S, James C, Mani J, Moffet M, Ougham H, Roberts L, Thomas A, Weeden N, Thomas H, King I (2007) Cross-species identification of Mendel's *I* locus. Science 315:73-73

Balazadeh S, Kwasniewski M, Caldana C, Mehrnia M, Zanor MI, Xue GP, Mueller-Roeber B (2011) ORS1, an H_2O_2-responsive NAC transcription factor, controls senescence in *Arabidopsis thaliana*. Mol. Plant 4:346-360

Barry CS, McQuinn RP, Chung MY, Besuden A, Giovannoni JJ (2008) Amino acid substitutions in homologs of the STAY-GREEN protein are responsible for the green-flesh and chlorophyll retainer mutations of tomato and pepper. Plant Physiol. 147:179-187

Delmas F, Sankaranarayanan S, Deb S, Widdup E, Bournonville C, Bollier N, Northey JGB, McCourt P, Samuel MA (2013) ABI3 controls embryo degreening through Mendel's *I* locus. Proc. Natl. Acad. Sci. USA 110:3888-3894

Fang C, Li C, Li W, Wang Z, Zhou Z, Shen Y, Wu M, Wu Y, Li G, Kong LA, Liu C, Jackson SA, Tian Z (2014) Concerted evolution of *D1* and *D2* to regulate chlorophyll degradation in soybean. Plant J. 77:700-712

Garapati P, Xue GP, Munne-Bosch S, Balazadeh S (2015) Transcription factor ATAF1 in *Arabidopsis* promotes senescence by direct regulation of key chloroplast maintenance and senescence transcriptional cascades. Plant Physiol. DOI:10.1104/pp.00567.2015

Grossman AR, Bhaya D, Apt KE, Kehoe DM (1995) Light-harvesting complexes in oxygenic photosynthesis: diversity, control, and evolution. Annu. Rev. Genet. 29:231-288

Guo Y, Gan S (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J. 46:601-612

Horie Y, Ito H, Kusaba M, Tanaka R, Tanaka A (2009) Participation of chlorophyll *b* reductase in the initial step of the degradation of light-harvesting chlorophyll *a/b*-protein complexes in *Arabidopsis*. J. Biol. Chem. 284:17449-17456

Hörtensteiner S (2013) Update on the biochemistry of chlorophyll breakdown. Plant Mol. Biol. 82:505-517

Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, Hwang D, Nam HG (2009) Trifurcate feed-forward regulation of age-dependent cell death involving *miR164* in *Arabidopsis*. Science 323:1053-1057

Kim YS, Sakuraba Y, Han SH, Yoo SC, Paek NC (2013) Mutation of the *Arabidopsis* NAC016 transcription factor delays leaf senescence. Plant Cell Physiol. 54:1660-1672

Kusaba M, Ito H, Morita R, Iida S, Sato Y, Fujimoto M, Kawasaki S, Tanaka R, Hirochika H, Nishimura M, Tanaka A (2007) Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. Plant Cell 19:1362-1375

Le DH, Kwon YK (2013) A coherent feed-forward loop design principle to sustain robustness of biological networks. Bioinformatics 29:630-637

Liang C, Wang Y, Zhu Y, Tang J, Hu B, Liu L, Ou S, Wu H, Sun X, Chu J, Chu C (2014) OsNAP connects abscisic acid and leaf senescence by fine-tuning abscisic acid biosynthesis and directly targeting senescence-associated genes in rice. Proc. Natl. Acad. Sci. USA 111:10013-10018

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 148:350-382

Mecey C, Hauck P, Trapp M, Pumplin N, Plovanich A, Yao J, He SY (2011) A critical role of STAYGREEN/Mendel's *I* locus in controlling disease symptom development during *Pseudomonas syringae* pv tomato infection of *Arabidopsis*. Plant Physiol. 157:1965-1974

Meguro M, Ito H, Takabayashi A, Tanaka R, Tanaka A (2011) Identification of the 7-hydroxymethyl chlorophyll *a* reductase of the chlorophyll cycle in *Arabidopsis*. Plant Cell 23:3442-3453

Nakajima S, Ito H, Tanaka R, Tanaka A (2012) Chlorophyll *b* reductase plays an essential role in maturation and storability of *Arabidopsis* seeds. Plant Physiol. 160:261-273

Olsen AN, Ernst HA, Leggio LL, Skriver K (2005) NAC transcription factors: structurally distinct, functionally diverse. Trends Plant Sci. 10:79-87

Parcy F, Valon C, Kohara A, Misera S, Giraudat J (1997) The ABSCISIC ACID-INSENSITIVE3, FUSCA3, and LEAFY COTYLEDON1 loci act in concert to control multiple aspects of *Arabidopsis* seed development. Plant Cell 9:1265-1277

Park SY, Yu JW, Park JS, Li J, Yoo SC, Lee NY, Lee SK, Jeong SW, Seo HS, Koh HJ, Jeon JS, Park YI, Paek NC (2007) The senescence-induced staygreen protein regulates chlorophyll degradation. Plant Cell 19:1649-1664

Pružinská A, Anders I, Aubry S, Schenk N, Tapernoux-Luthi E, Muller T, Krautler B, Hörtensteiner S (2007) In vivo participation of red chlorophyll catabolite reductase in chlorophyll breakdown. Plant Cell 19:369-387

Pružinská A, Tannerm G, Anders I, Roca M, Hörtensteiner S (2003) Chlorophyll breakdown: pheophorbide *a* oxygenase is a Rieske-type iron-sulfur protein, encoded by the *accelerated cell death 1* gene. Proc. Natl. Acad. Sci. USA 100:15259-15264

Rauf M, Arif M, Dortay H, Matallana-Ramirez LP, Waters MT, Nam HG, Lim PO, Mueller-Roeber B, Balazadeh S (2013) ORE1 balances leaf senescence against maintenance by antagonizing G2-like-mediated transcription. EMBO Rep. 14:382-388

Ren G, An K, Liao Y, Zhou X, Cao Y, Zhao H, Ge X, Kuai B (2007) Identification of a novel chloroplast protein AtNYE1 regulating chlorophyll degradation during leaf senescence in Arabidopsis. Plant Physiol. 144:1429-1441

Sakuraba Y, Jeong J, Kang MY, Kim J, Paek NC, Choi G (2014c) Phytochrome-interacting transcription factors PIF4 and PIF5 induce leaf senescence in *Arabidopsis*. Nat. Commun. 5:4636

Sakuraba Y, Kim D, Kim YS, Hörtensteiner S, Paek NC (2014b) *Arabidopsis* STAYGREEN-LIKE (SGRL) promotes abiotic stress-induced leaf yellowing during vegetative growth. FEBS Lett. 588:3830-3837

Sakuraba Y, Kim YS, Han SH, Lee BD, Paek NC (2015b) The *Arabidopsis* transcription factor NAC016 promotes drought stress responses by repressing *AREB1* transcription through a trifurcate feed-forward regulatory loop involving NAP. Plant Cell. DOI:10.1105/tpc.15.00222

Sakuraba Y, Kim YS, Yoo SC, Hörtensteiner S, Paek NC (2013) 7-Hydroxymethyl chlorophyll *a* reductase functions in metabolic channeling of chlorophyll breakdown intermediates during leaf senescence. Biochem. Biophys. Res. Commun. 430:32-37

Sakuraba Y, Park SY, Kim YS, Wang SH, Yoo SC, Hörtensteiner S, Paek NC (2014a) *Arabidopsis* STAY-GREEN2 is a negative regulator of chlorophyll degradation during leaf senescence. Mol. Plant 7:1288-1302

Sakuraba Y, Park SY, Paek NC (2015a) The divergent roles of STAYGREEN (SGR) homologs in chlorophyll degradation. Mol. Cells 38:390-395

Sakuraba Y, Schelbert S, Park SY, Han SH, Lee BD, Andres CB, Kessler F, Hörtensteiner S, Paek NC (2012) STAY-GREEN and chlorophyll catabolic enzymes interact at light-harvesting complex II for chlorophyll detoxification during leaf senescence in *Arabidopsis*. Plant Cell 24:507-518

Saleh A, Alvarez-Venegas R, Avramova Z (2008) An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in *Arabidopsis* plants. Nat. Protoc. 3:1018-1025

Sato Y, Morita R, Katsuma S, Nishimura M, Tanaka A, Kusaba M (2009) Two short-chain dehydrogenase/reductases, NON-YELLOW COLORING 1 and NYC1-LIKE, are required for chlorophyll *b* and light-harvesting complex II degradation during senescence in rice. Plant J. 57:120-131

Schelbert S, Aubry S, Burla B, Agne B, Kessler F, Krupinska K, Hörtensteiner S (2009) Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in *Arabidopsis*. Plant Cell 21:767-785

Wei Q, Guo Y, Kuai B (2011) Isolation and characterization of a chlorophyll degradation regulatory gene from tall fescue. Plant Cell Rep. 30:1201-1207

Wu A, Allu AD, Garapati P, Siddiqui H, Dortay H, Zanor MI, Asensi-Fabado MA, Munné-Bosch S, Antonio C, Tohge T, Fernie AR, Kaufmann K, Xue GP, Mueller-Roeber B, Balazadeh S (2012) *JUNGBRUNNEN1*, a reactive oxygen species-responsive NAC transcription factor, regulates longevity in *Arabidopsis*. Plant Cell 24:482-506

Yang J, Worley E, Udvardi M (2014) A NAP-AAO3 regulatory module promotes chlorophyll degradation via ABA biosynthesis in *Arabidopsis* leaves. Plant Cell 26:4862-4874

Yang SD, Seo PJ, Yoon HK, Park CM (2011) The *Arabidopsis* NAC transcription factor VNI2 integrates abscisic acid signals into leaf senescence via the *COR/RD* genes. Plant Cell 23:2155-2168

Zhang K, Gan SS (2012) An abscisic acid-AtNAP transcription factor-SAG113 protein phosphatase 2C regulatory chain for controlling dehydration in senescing *Arabidopsis* leaves. Plant Physiol. 158:961-969

Zhou C, Han L, Pislariu C, Nakashima J, Fu C, Jiang Q, Quan L, Blancaflor EB, Tang Y, Bouton JH, Udvardi M, Xia G, Wang ZY (2011) From model to crop: functional analysis of a *STAY-GREEN* gene in the model legume *Medicago truncatula* and effective use of the gene for alfalfa improvement. Plant Physiol. 157:1483-1496

Fig. 1. The altered expression of *CCE* genes in *nac016-1* mutants during DIS.
The 3-week-old WT (black bars) and *nac016-1* (white bars) grown under long day (16 h light:8 h dark) conditions were dark-incubated and rosette leaves were sampled at 0 and 4 DDI for RT-qPCR analysis. Relative transcript levels of *NYC1* (a), *NOL* (b), *HCAR* (c), *PPH* (d), *PAO* (e), *RCCR* (f), *SGR1* (g), *SGR2* (h), and *SGRL* (i) in WT and *nac016-1* plants at 0 and 4 DDI were normalized to the transcript levels of *GAPDH*. Mean and SD values were obtained from more than three replicates. Asterisks indicate significant difference compared with WT (Student's *t*-test *P* values, **P* < 0.05, ***P* < 0.01). These experiments were repeated twice with similar results. DDI, days of dark incubation.

Fig. 2. NAC016 directly binds to the *SGR1* promoter.
(a) The NAC016BM sites in the promoters of *SGR1*, *SGR2*, and *NYC1* are shown. Blue and green horizontal lines indicate the DNA fragments used for yeast one-hybrid (b) and ChIP assays (c), respectively. (b) Analysis of the binding activity of NAC016 (N16) to the promoter regions of *SGR1* (*SGR1*-I and *SGR1*-II), *SGR2* (*SGR2*-I and *SGR2*-II), and *NYC1* (*NYC1*-I and *NYC1*-II) in yeast one-hybrid assays. β-Galactosidase (β-Gal) activity (1 unit = 10^4 min^{-1} ml^{-1}) was measured by liquid assays using CPRG. Empty bait and prey plasmids (-) were used for negative controls. Asterisks indicate significant difference (Student's *t*-test *P* value, **P* < 0.05, ***P* < 0.01). NS, not significant. (c) NAC016 binding affinity to the promoter regions of *SGR1*, *SGR2*, and *NYC1 in planta*. Fold enrichment of the promoter fragments of *SGR1*, *SGR2*, and *NYC1* was measured by ChIP assay with an anti-GFP antibody (see Methods). Two-week-old WT and *GFP-NAC016* plants at 2 DDI were used. *TUB2* was used as a negative control. Asterisks indicate significant difference compared with *TUB2* (Student's *t*-test *P* values, ***P* < 0.01). These experiments (b, c) were repeated twice with similar results.

Fig. 3. Altered expression of *SGR1* in *nac016-1* and *NAC016*-OX during senescence.
The relative expression levels of *SGR1* were measured and normalized to the transcript levels of *GAPDH* during DIS (a), natural senescence (b), and salt treatment (c). (a) For DIS, the 3-week-old WT and *nac016-1* plants grown under LD (16 h light:8 h dark) conditions were dark-incubated at 22°C. For salt treatment, rosette leaves detached from 3-week-old plants grown under LD conditions were floated on 3 mM MES buffer (pH 5.7) supplemented with 100 mM NaCl at 22°C. Mean and SD values were obtained from more than three replicates. Asterisks indicate significant difference compared with WT (Student's *t*-test *P* value, **P* < 0.05, ***P* < 0.01). These experiments

were repeated twice with similar results. DDI, days of dark incubation. WAG, weeks after germination. HT, hours of treatment.

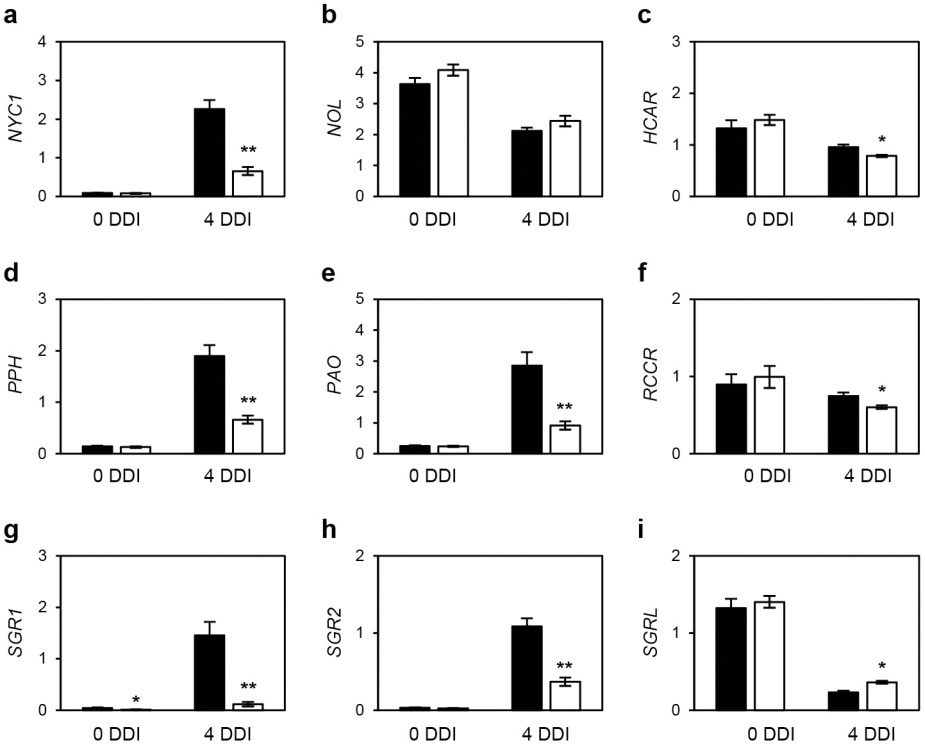
Fig. 4. NAC016 acts genetically upstream of *SGR1*.
The changes of leaf color (a) and total Chl levels (b) in WT, *SGR1*-OX, *nac016-1*, and *nac016-1 SGR1*-OX plants at 0 and 4 DDI. The rosette leaves detached from 3-week-old plants grown under LD conditions were floated on the 3 mM MES buffer (pH 5.7) and incubated in complete darkness. (b) Black and white bars indicate 0 and 4 DDI, respectively. Mean and SD values were obtained from more than five replicates. Asterisks indicate significant difference compared with WT (Student's *t*-test *P* value, $*P < 0.05$, $**P < 0.01$). These experiments were repeated twice with similar results.

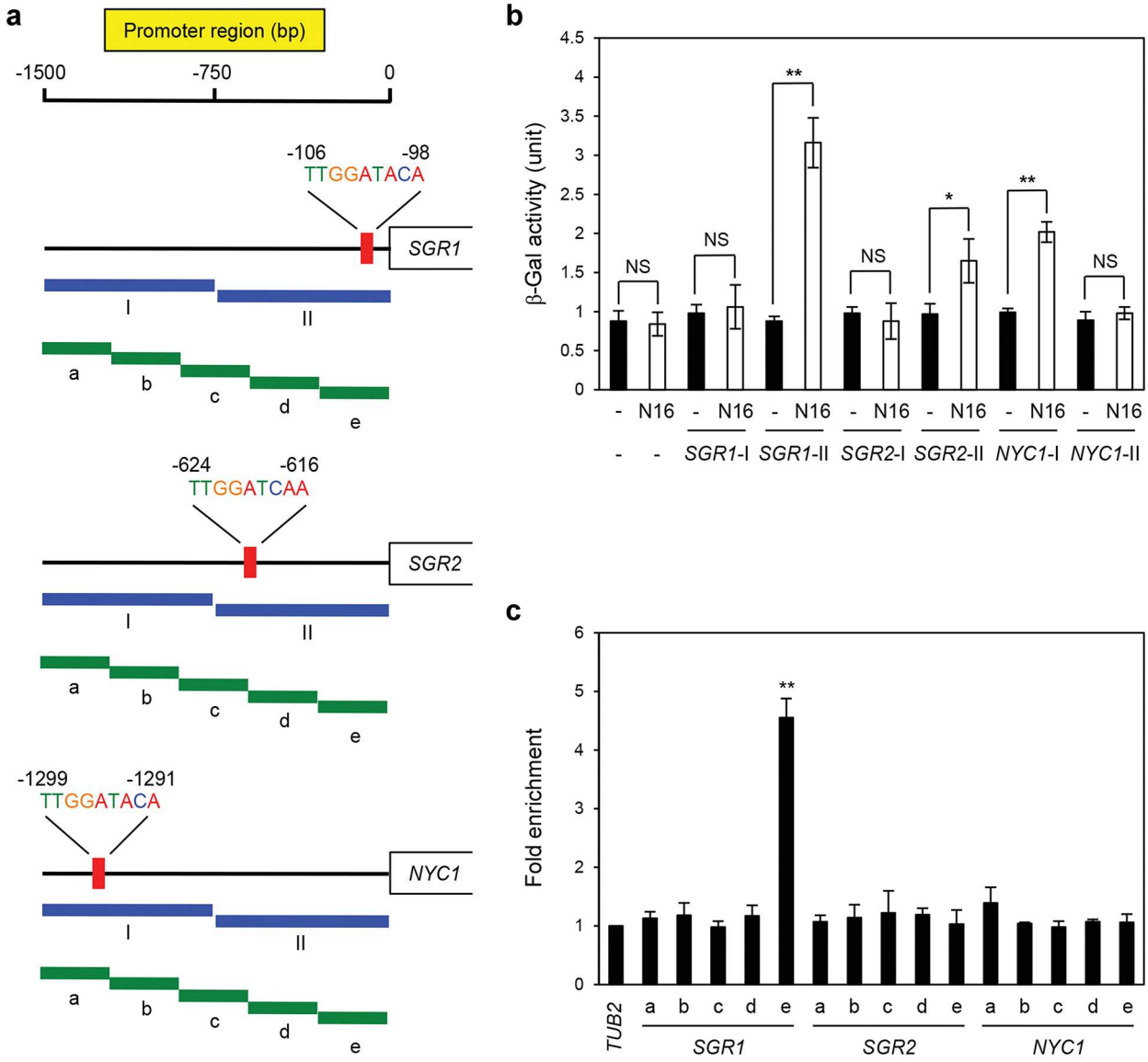
Fig. 5. *nac016-1* mutants differ from *nye1-1* mutants during seed degreening.
(a-d) Visible phenotypes (a), Chl autofluorescence (b), total Chl levels (c), and germination rates (d) of the WT, *nye1-1*, and *nac016-1* seeds. (b) Chl autofluorescence in *Arabidopsis* seeds was observed by confocal microscopy. (c) Total Chl levels were quantified using 500 seeds in each sample. (e) The color difference of WT, *nye1-1*, and *nac016-1* seedlings at the cotyledon stage. (f) The percentage of albino phenotype of WT, *nye1-1*, and *nac016-1* seedlings at the cotyledon stage. (g) Relative expression levels of *SGR1* in mature WT and *nac016-1* seeds was determined by RT-qPCR analysis, and normalized to the transcript levels of *GAPDH*. (c, d, f, g) Mean and SD values were obtained from more than three replicates. Asterisks indicate significant difference compared with WT (Student's *t*-test *P* value, $*P < 0.05$, $**P < 0.01$). These experiments were repeated twice with similar results.

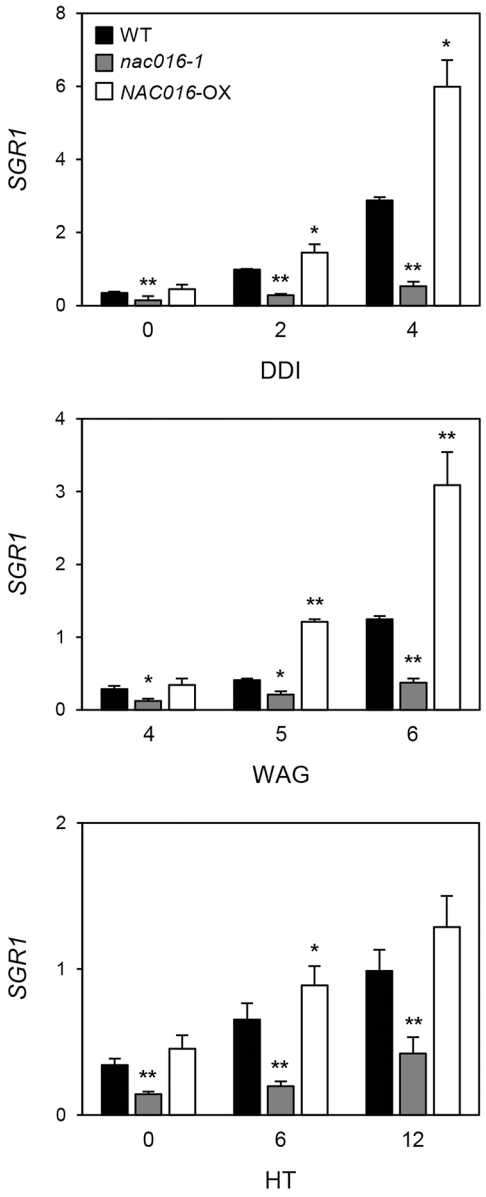
Fig. 6. Altered expression of *NAP*, *AAO3*, *ABI5*, and *EEL* in *nac016-1* and *NAC016*-OX plants during DIS and natural senescence.
Relative expression levels of *NAP* (a, b), *AAO3* (c, d), *ABI5* (e, f), and *EEL* (g, h) during DIS (a, c, e, g) and natural senescence (b, d, f, h) were determined by RT-qPCR analysis and normalized to the transcript level of *GAPDH*. Mean and SD values were obtained from more than three replicates. Asterisks indicate significant difference compared with WT (Student's *t*-test *P* value, $*P < 0.05$, $**P < 0.01$). These experiments were repeated twice with similar results.

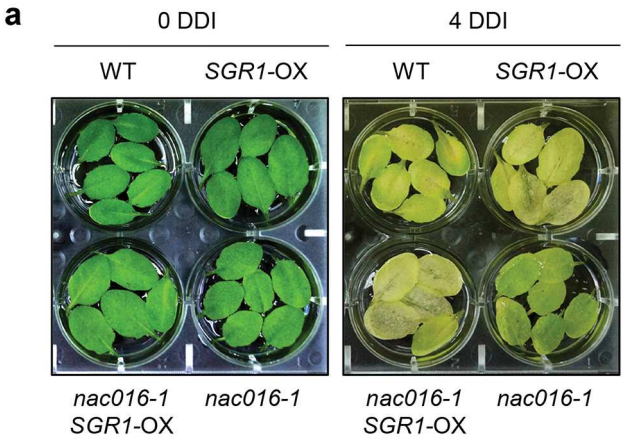
Fig. 7. Model of NAC016-mediated regulation of *SGR1* and *NYC1* in the Chl degradation pathway.

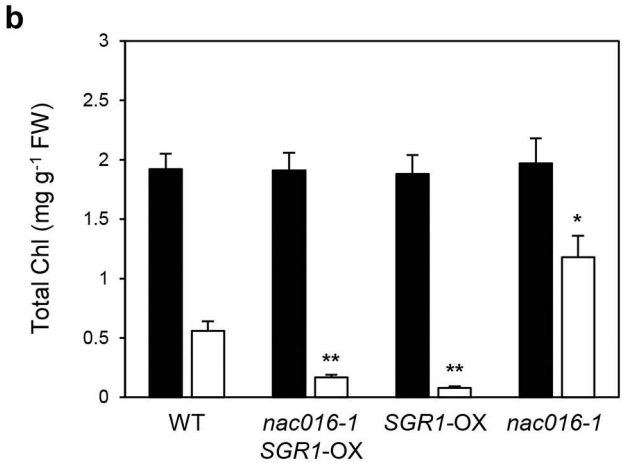
Black solid lines indicate the regulation that was previously identified. The red solid line indicates the findings in this study. Black dotted lines indicate the unidentified regulation that can be expected.

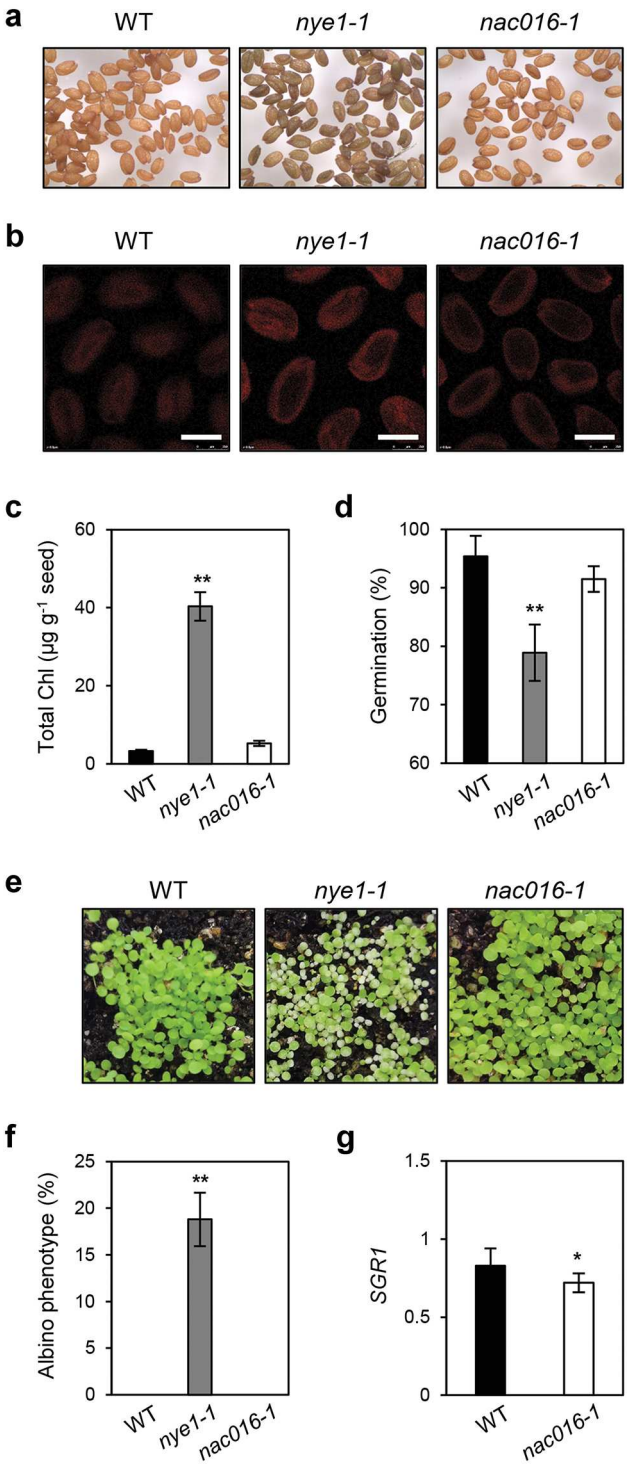

a
NYC1
0 DDI
4 DDI
b
NOL
0 DDI
4 DDI
c
HCAR
0 DDI
4 DDI
d
PPH
0 DDI
4 DDI
e
PAO
0 DDI
f
RCCR
0 DDI
4 DDI
g
SGR1
0 DDI
4 DDI
h
SGR2
0 DDI
4 DDI
i
SGRL
0 DDI
4 DDI

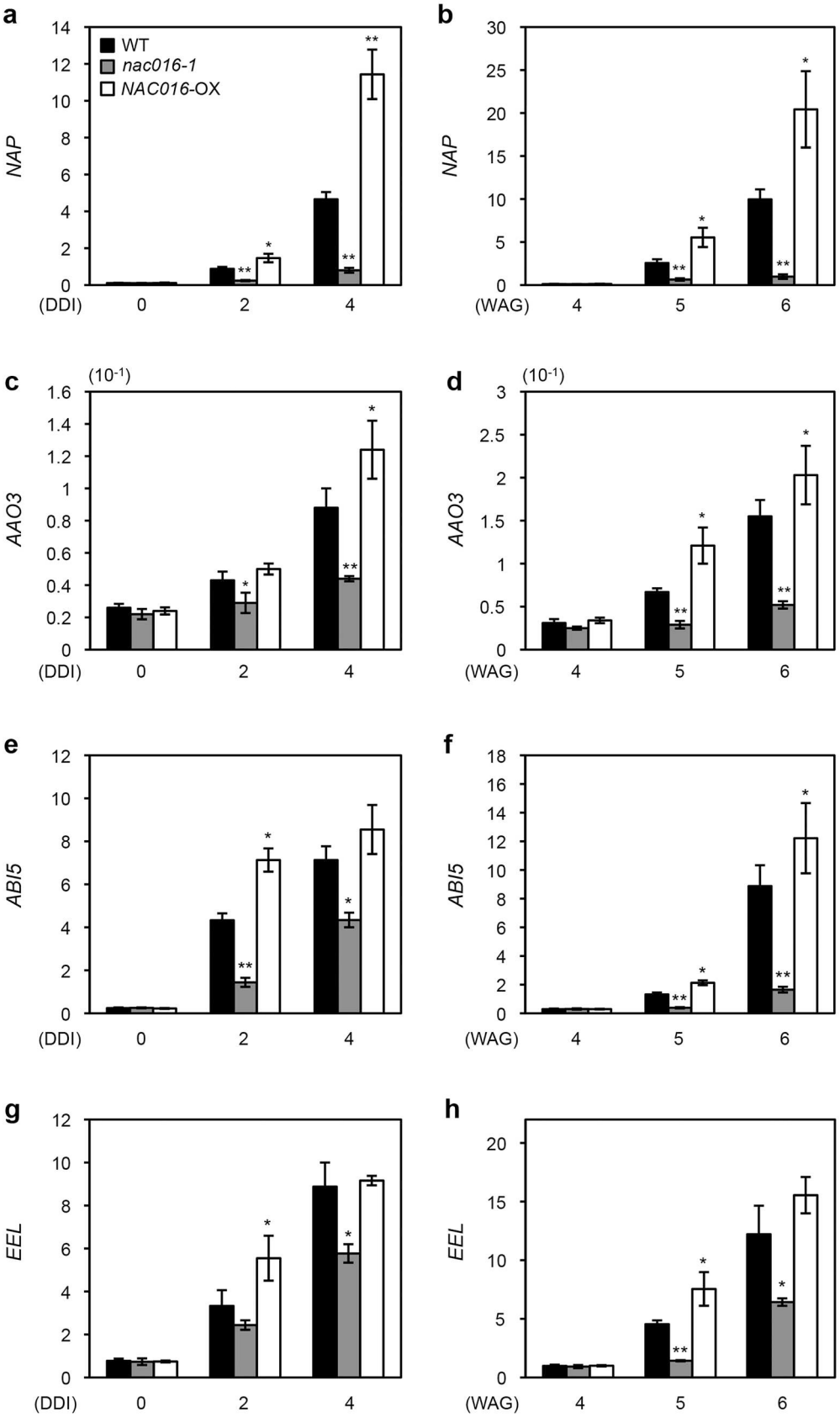

a
Promoter region (bp)
-1500
-750
0
-106
-98
TTGGATACA
SGR1
I
II
a
b
c
d
e
-624
-616
TTGGATCAA
SGR2
I
II
a
b
c
d
e
-1299
-1291
TTGGATACA
NYC1
I
II
a
b
c
d
e
b
β-Gal activity (unit)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
*
**
NS
NS
NS
NS
NS
- N16 - N16 - N16 - N16 - N16 - N16 - N16
- - SGR1-I SGR1-II SGR2-I SGR2-II NYC1-I NYC1-II
c
Fold enrichment
6
5
4
3
2
1
0
**
TUB2
a b c d e a b c d e a b c d e
SGR1
SGR2
NYC1

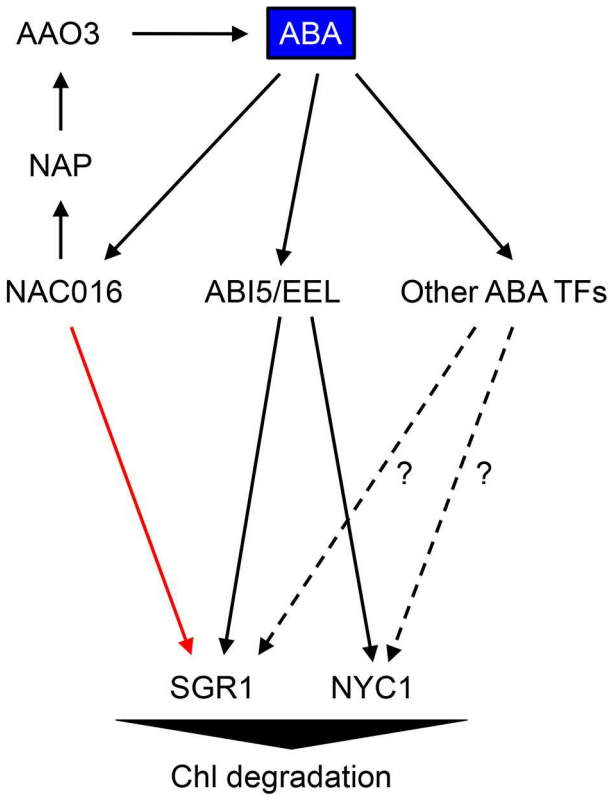

WT
nac016-1
NAC016-OX
SGR1
DDI
0
2
4
WAG
4
5
6
HT
0
6
12


a
0 DDI
4 DDI
WT
SGR1-OX
WT
SGR1-OX
nac016-1 SGR1-OX
nac016-1
nac016-1 SGR1-OX
nac016-1

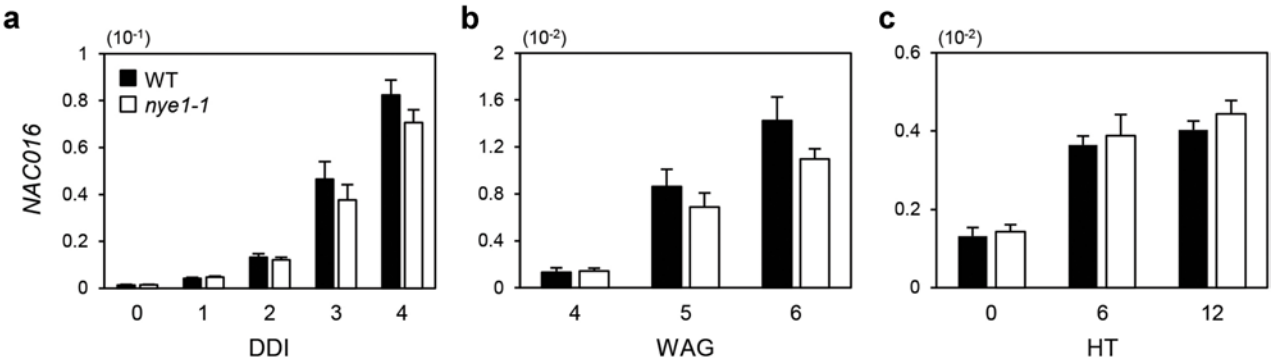

b
Total Chl (mg g-1 FW)
3
2.5
2
1.5
1
0.5
0
**
**
*
WT
nac016-1 SGR1-OX
SGR1-OX
nac016-1


a
WT
nye1-1
nac016-1
b
WT
nye1-1
nac016-1
c
Total Chl (μg g-1 seed)
60
40
20
0
**
WT
nye1-1
nac016-1
d
Germination (%)
100
90
80
70
60
**
WT
nye1-1
nac016-1
e
WT
nye1-1
nac016-1
f
Albino phenotype (%)
25
20
15
10
5
0
**
WT
nye1-1
nac016-1
g
SGR1
1.5
1
0.5
0
*
WT
nac016-1

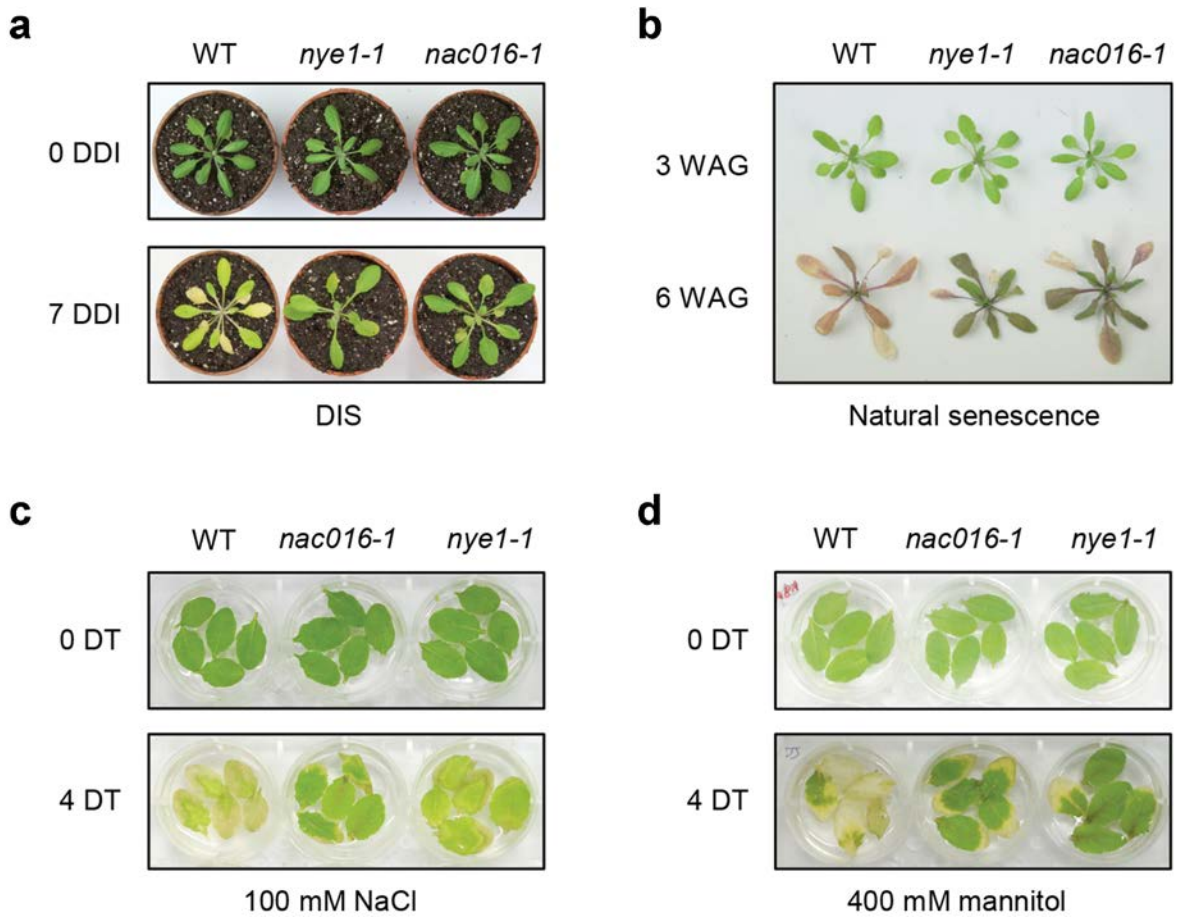

a
b
c
d
e
f
g
h
WT
nac016-1
NAC016-OX
NAP
AAO3
ABI5
EEL
(DDI)
(WAG)
(10-1)


AAO3
ABA
NAP
NAC016
ABI5/EEL
Other ABA TFs
?
?
SGR1
NYC1
Chl degradation

Supplementary Information - Sakuraba et al. (2015)

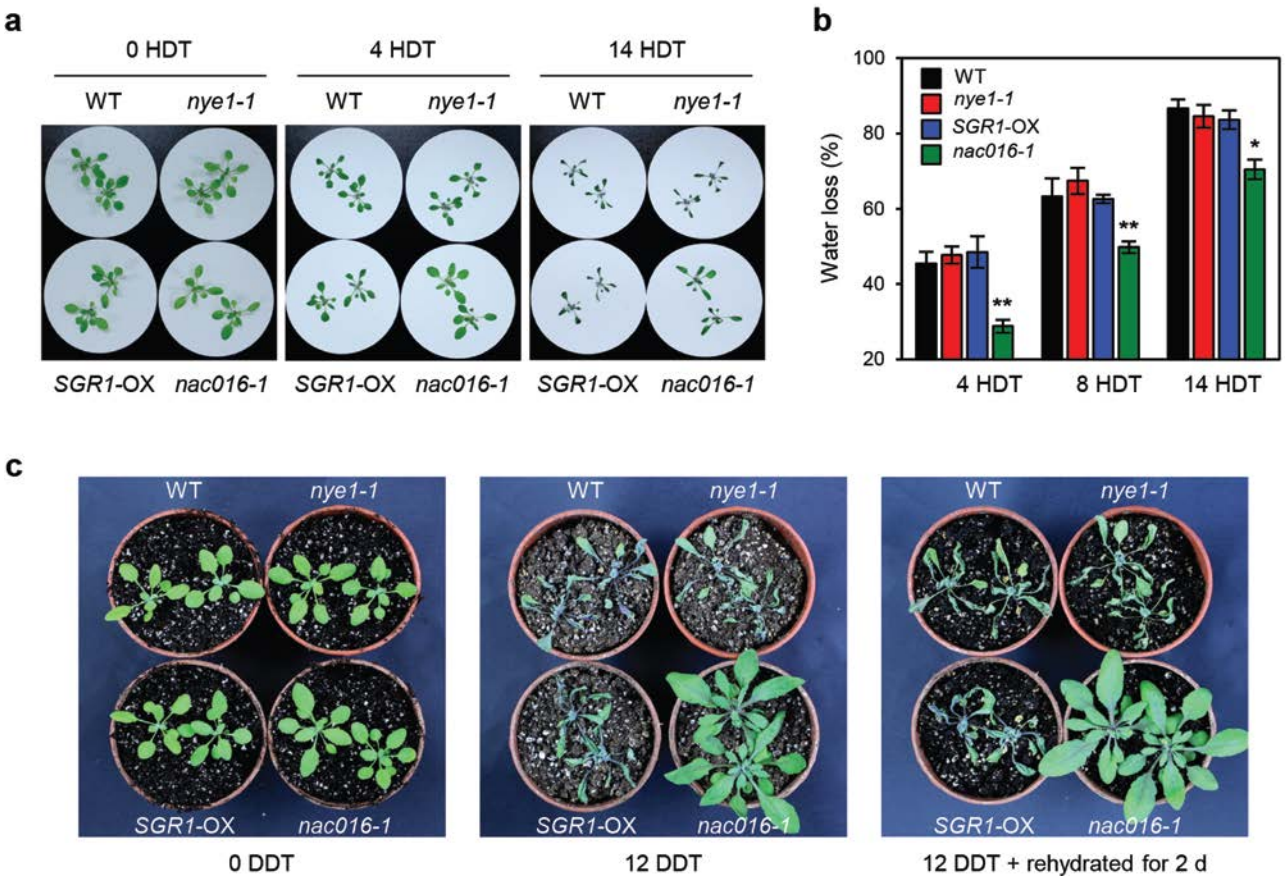


Supplementary Fig. S1. Altered expression of *NAC016* during leaf senescence.
Relative expression level of *NAC016* during DIS (A), natural senescence (B), and salt-induced senescence (C) in WT and *nye1-1* mutants (white bars) was determined by RT-qPCR analysis and normalized to the transcript levels of *GAPDH*. Mean and SD values were obtained from more than three replicates. These experiments were repeated twice with similar results. DDI, day(s) of dark incubation. WAG, week(s) after germination. HT, hour(s) of treatment.

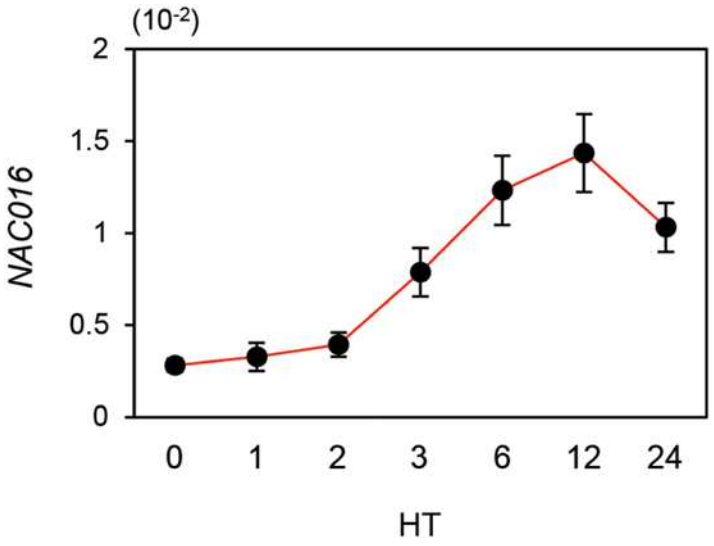


Supplementary Fig. S2. *nye1-1* and *nac016-1* mutants showed stay-green phenotypes during DIS, natural senescence, and stress-induced senescence.

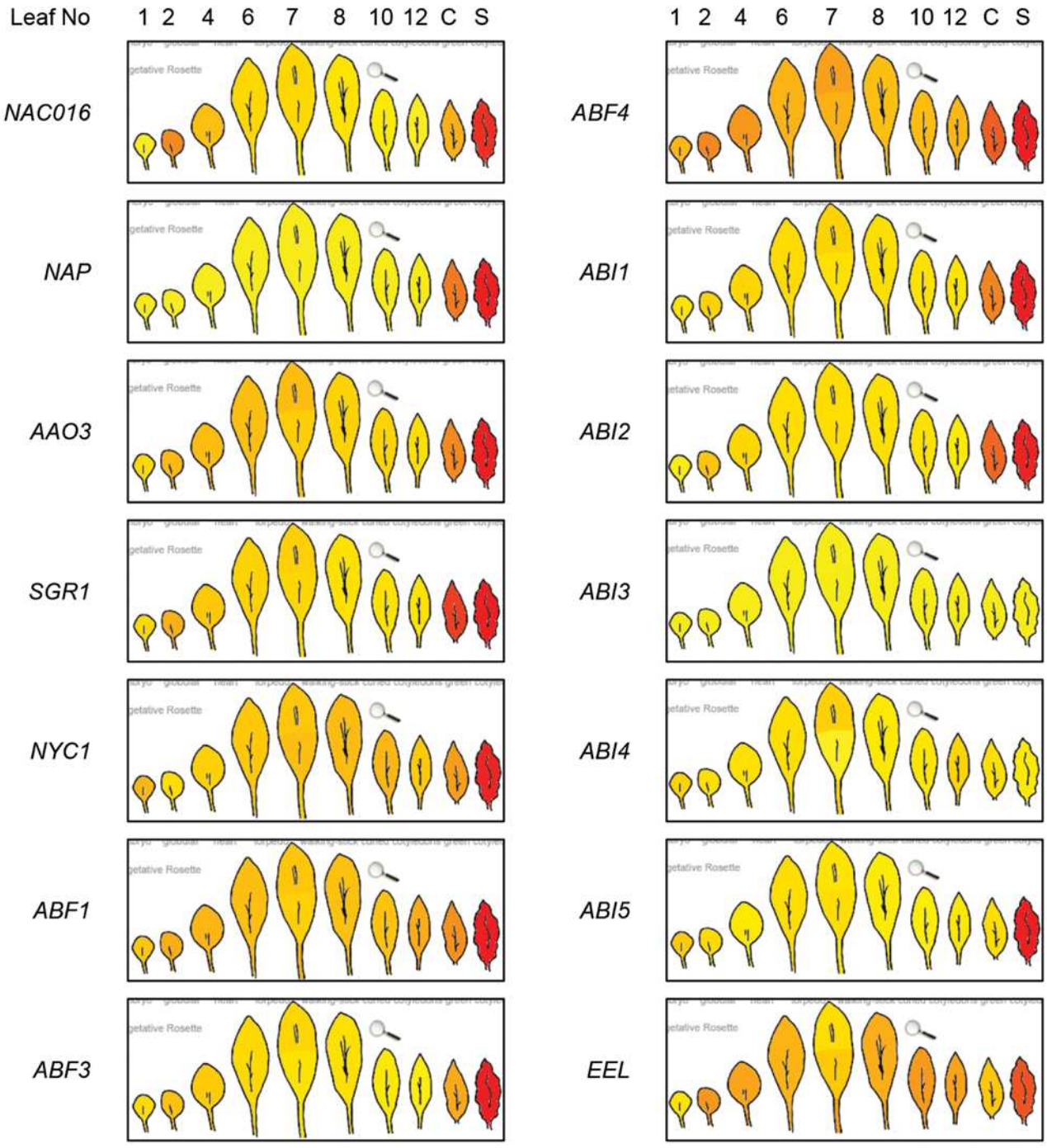
The phenotypes of WT, *nye1-1*, and *nac016-1* mutants at 0 DDI and 7 DDI (A), natural senescence (B), salt-induced senescence (C), and osmotic stress-induced senescence (D) were shown. DDI, day(s) of dark incubation; WAG, weeks after germination; DT, day(s) of treatment.



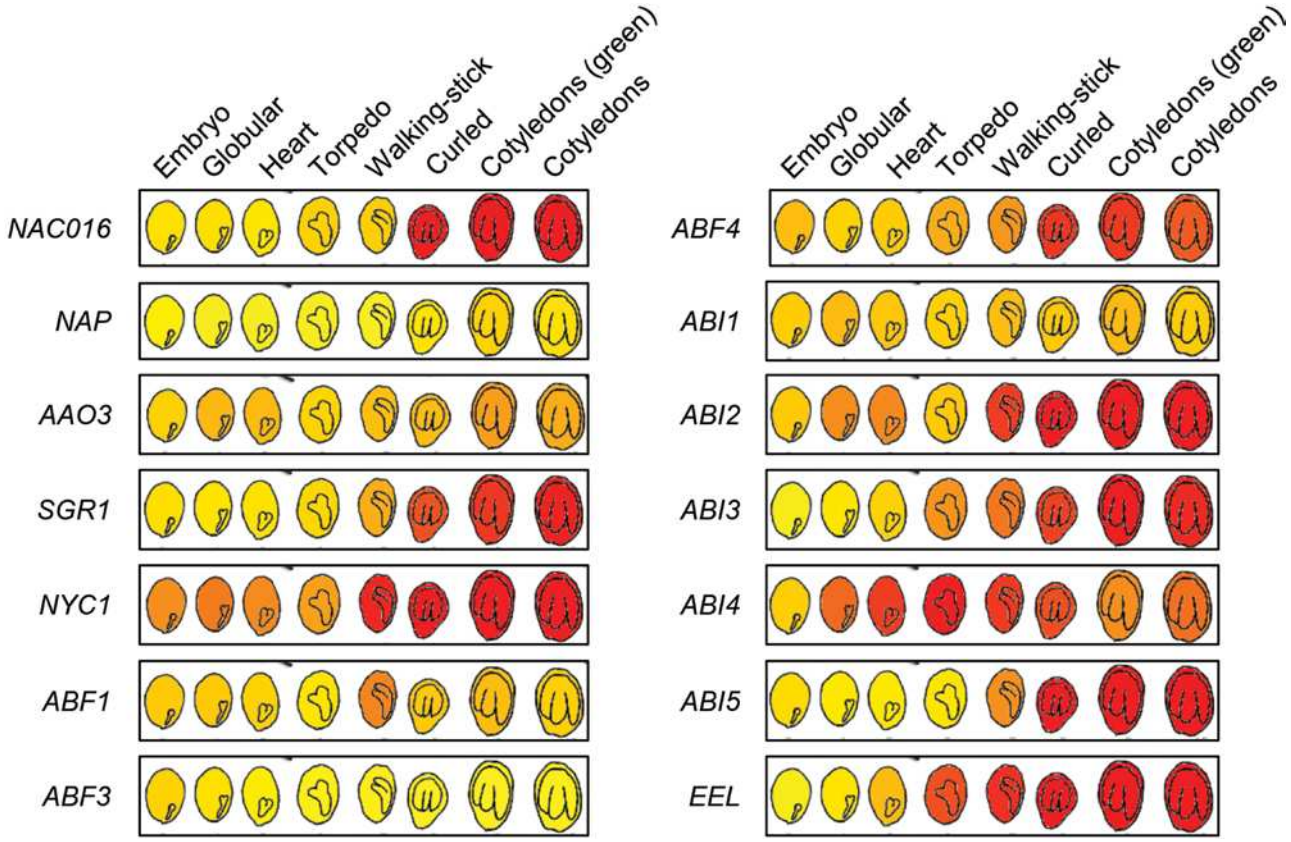
Supplementary Fig. S3. Drought stress assays on *nye1-1* and *SGR1*-OX plants.
(a) The 3-week-old WT, *nye1-1*, *SGR1*-OX, and *nac016-1* (positive control) plants were placed on dry filter paper and dehydrated for 14 h. (b) Water loss rate was calculated at 4, 8, and 14 HDT. Black, red, blue, and green bars indicate WT, *nye1-1*, *SGR1*-OX, and *nac016-1*, respectively. Asterisks indicate significant difference compared with WT (Student's *t*-test *P* value, * $P < 0.05$, ** $P < 0.01$). (c) Drought stress assay on soil. Three-week-old plants were dehydrated for 12 days (12 DDT, middle panel) and then rehydrated for 2 days (right panel). HDT, hours of drought treatment; DDT, days of drought treatment.



Supplementary Fig. S4. The expression of *NAC016* in response to exogenous ABA treatment. Rosette leaves detached from the 3-week-old WT plants grown under LD conditions were floated on 3 mM MES buffer (pH 5.7) supplemented with 50 μM ABA, and incubated under continuous light conditions. Mean and SD values were obtained from more than three replicates. These experiments were repeated twice with similar results. HT, hour(s) of treatment.



Supplementary Fig. S5. The expression patterns of *NAC016*, *NAP*, *AAO3*, *SGR1*, *NYC1*, and ABA signaling-related genes *ABF1* and *ABF3* in leaves of different ages.
These data were obtained from the *Arabidopsis* eFP browser website (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).



Supplementary Fig. S6. The expression patterns of *NAC016*, *NAP*, *AAO3*, *SGR1*, *NYC1*, and ABA signaling-related genes *ABF1* and *ABF3* in different growth stage of seeds. These data were obtained from the *Arabidopsis* eFP browser website (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

Supplementary Table S1. Primer information used in this study.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
(A) RT-qPCR		
NYC1	GTTAACAGACGCGATGGAGA	GCCTGGAAAAGAGCTAGGTG
NOL	TGCAGATGCAAGATGTCAAA	TGGTGTAGGCTTTGATTCCA
HCAR	CGGTACTCGTTGGACTACCAT	CTATTTGGCCGTTTTTTGTTGT
PPH	TCTCACGTATTGTGGAGGTC	ATAGCTCTCCACCAGGAGCA
PAO	CTCTGGTTTGATCGGAATGAT	GAAGCTCGTGCTGTTAAATCC
RCCR	CGCCGAAAATTTATGGAGTT	AGGGAAGGAGTTGTGATTGG
SGR1	TGGGCAAATAGGCTATACCG	CCACCGCTTATGTGACAATG
SGR2	TCCCGGAGTACAACAAGGTC	TTGCACTCATCAGGACAAGG
SGRL	TTGCTGAGTGGAAGAAGGTG	ACCTAAGCTCAGCAGCAACA
AAO3	GAAGGTCTTGGAAACACGAAGAA	GAAATACACATCCCTGGTGTACAAA
ABI5	CAGCAAATGGGAATGGTTG	TCTCCACTGGACCATCCAC
EEL	TTTGTTTTGGGGAGCATCA	TCGGTTCAACAGTGAGTTGC
GAPDH	TTGGTGACAACAGGTCAAGCA	AAACTTGTCGCTCAATGCAATC
(B) Yeast one-hybrid assay		
SGR1-I	GAATTCTAGTTGACTTTAACAGACGCATAG	GTCGACAAGGGCTGAAATTTGT
SGR1-II	GAATTCTTAACATCCTTATGGATCAA	GTCGACCTCTGCTCTCTTGAA
SGR2-I	GAATTCCCCATATATCACGCA	GTCGACGTTAGATTAATGTAAAAA
SGR2-II	GAATTCCAGTTAATTTTTACATTAATC	GTCGACCTTTGCTTGTTCTCA
NYC1-I	GTCGACCCAAGCCCTAGGTC	CTCGAGAAATTTGGACCCTTTTTTA
NYC1-II	GAATTCAACCATCTGATTAGTATGGAA	GTCGACAGCTATGGAAGAAGAGTGAG
(C) ChIP assay		
SGR1-a	TAGTTGACTTTAACAGACGCATAG	GCGAAATCTTTTGATGATGATT
SGR1-b	CTATTCAATCATCATCAAAAGATTTC	AAACACGAGTACTGTTTTTTCTTCTTC
SGR1-c	GAGATTGAAGGAGAAAGAAGAAGAA	ATTACTCGGTTTAGTTATTAGACCTGTG
SGR1-d	CGAGTCGAATGACATTAAATTGAA	TTGTGTTTAAAAGGGATTGTTGC
SGR1-e	GAACCAACTCAATACGCATCACA	CTCTGCTCTCTTGAAACCCAAA
SGR2-a	CCCATATATCACGCAGATTTTTC	GTTATTAAGTAACGTCCCTCCAAA
SGR2-b	GGTTTGGAGGGACGTTACTTAATAA	GCAAATTGTAGAAATATCTAAACTAATTTAC
SGR2-c	GTAAATTAGTTTAGATATTTCTACAATTTGC	GAGTTAACAATGATTGATCCAACTCG
SGR2-d	CAATGTATTTTTGAGAAATTAACCGA	TCAAAGATCTCTTTGAACAGGTGAG
SGR2-e	GAGATCTTTGAAGAACTGAGAAGAA	CTTTGCTTGTTCTCAAAAATATCC
NYC1-a	CCAAGCCCTAGGTCCAGTG	AACAACCCTTTCCCCCTCTTT
NYC1-b	CAAATCAATAAAAGAGGGGGAAA	GAGCCGGTCTGCTTTACGT
NYC1-c	GTCTCACGTAAAGCGAGACCG	ATATCAAACAAAGAAAAGTTGATAATG
NYC1-d	GCTTAAAGTTTACACAAAGGAGAAAA	CTTTTGTCCCTTCGCATGTTTT
NYC1-e	GAAGGGCAAAAGAAACACAAA	AGCTATGGAAGAAGAGTGAGATAATGAT